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# Recombinant LipL32 Protein for Leptospirosis Detection in Indonesia

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**Abstract**

Leptospirosis is an endemic zoonotic disease in tropical countries. However, detection using the microagglutination test (MAT) as a gold standard is difficult to perform in Indonesia. Therefore, recombinant LipL32 protein (rLipL32) has been studied as an antigen for an ELISA test to detect Leptospirosis. We produced rLipL32 using the pRSET-C vector and expressed it in *E. coli* BL21 (DE3) competent cells. Under native conditions, we purified 2 mg rLipL32 protein from 500 ml culture. Analysis using western blotting and ELISA showed that serum from the bovine positive MAT serovar Hardjo could recognize pure rLipL32 protein. This result confirms an earlier study that indicates that rLipL32 protein is a good antigen for Leptospirosis detection. The diagnostic assay using rLipL32 is safe because it does not use infectious bacteria as an antigen and because it is easy to perform in every diagnostic laboratory in Indonesia. However, further study is still required for field validation of the rLipL32 assay.


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*Keywords:* Leptospirosis ; LipL32; recombinant protein; ELISA

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## 1. Introduction

Leptospirosis is an important zoonotic disease that is caused by *Leptospira spirochaetes*. It is transmitted from animals or the environment to humans by direct or indirect contact. Clinical manifestations from infection by pathogenic *Leptospira* range from fever – flu like symptoms to severe cases with jaundice and fulminant - hepato renal failure[1,2]. Furthermore, effects on reproduction are of concern for livestock like cattle and pig[3].

Leptospirosis is commonly found in humid tropical and subtropical countries. A previous study in Indonesia reported high degrees of seropositivity of human and bovine serum to Leptospirosis. Diagnostic microagglutination tests during 2002-2004 showed seropositivity of human samples of around 10% to 11%. In contrast, seropositivity of bovine samples in 2002, 2003 and 2004 amounted to 24.65%, 7.41% and 17.38% respectively[4].

Leptospirosis diagnosis is generally done based on serological tests using a microagglutination test (MAT) as the gold standard[5,6]. However, the test has a high risk of infection to personnel because it uses life infectious bacteria as antigen[7]. Moreover, the antigen should be subcultured every week, which may cause problems in the standardization. The gold standard test for Leptospirosis (MAT) is also serovar specific and requires a complete panel of *Leptospira* serovars[3].

Because of many drawbacks, various serological tests were developed as alternatives for MAT and four of them have been evaluated, which were IgM-ELISA, an indirect hemagglutination assay, an IgM dipstick assay, and an IgM dot-ELISA dipstick test[8]. Determination and identification of protein antigens that are "conserved" in all serovar pathogens is also essential for production of single antigens in diagnostic tests for Leptospirosis. Recent researches were focused on the identification of outer membrane proteins (OMPs) of bacteria that can be used in the development of diagnostic tests for leptospirosis[9]. Several recombinant antigens have been produced including transmembrane protein (OMPL1), lipoprotein (LipL32, LipL36 and LipL41) and heat shock protein (HsP58) and used for development of ELISAs for diagnosis of leptospirosis in humans[10].

In this study we focus on LipL32 because it is the main protein of the outer membrane of *Leptospira* and as much as 40000 copies of LipL32 were found in each *Leptospira* cell[11]. This protein is conserved and expressed only in pathogenic *Leptospira* strains[12,13]. The LipL32 protein has been widely studied as a good candidate for a universal antigen to detect Leptospirosis[14,15]. However, application of LipL32 in an ELISA in Indonesia has not been reported. Therefore, this study aimed to produce recombinant LipL32 protein and use it as an antigen in an ELISA assay for Leptospirosis detection in bovine.

## 2. Methods

### *2.1. Amplification of the LipL32 gene*

DNA was extracted from 200 µl culture of *Leptospira* serovar Pomona using QIAamp DNA mini kit (Qiagen) and the concentration was measured using a Nanodrop instrument. The DNA was used as a template for amplification of the LipL32 gene by the Polymerase Chain Reaction (PCR), with forward primer LipL32-F and reverse primer LipL32-R2. The PCR reaction was performed in a total volume of 50 µl consisting of 9 µl nuclease-free water, 10 µl 5x reaction buffer, 10 µl 5x Q solution, 5 µl forward primer 10 µM, 5 µl reverse primer 10 µM, 1 µl HotstarQiagen High-Fidelity DNA Polymerase, and 10 µl *Leptospira* DNA (6.8 ng/µl). The thermal cycler was conditioned with one cycle of initial denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation for 15 seconds at 94°C, primer annealing for 1 minute at 63°C, and extension for 1 minute at 72°C, also 1 cycle for final extension for 5 minutes at 72°C. The PCR product was visualized by electrophoresis using 1% agarose gel to analyse the size and purity. The amplified LipL32 gene (size 782 bp) was confirmed by comparing the band with a DNA ladder (results not shown). The PCR product was purified using a gel extraction kit (Qiagen) and the concentration was measured using a Nanodrop instrument, and then stored at -20°C.

Table 1. Primer used in PCR LipL32 gene containing restriction site (underlined and italic).

| Name | Oligonucleotide | Restriction site | References |
|---|---|---|---|
| Forward primer: | | | |
| LipL32-F | 5'-TTA CCG *CTC GAG* GTG CTT TCG GTG GTC TGC-3' | *XhoI* | (16, 17) |
| Reverse primer: | | | |
| LipL32-R2 | 5'-TGT TAA *GAA TTC* TTA CTT AGT CGC GTC AGA-3' | *EcoRI* | (17) |

### *2.2. Digestion and ligation of plasmid pRSET-C and LipL32 gene*

Double digestion was performed using the *Eco*RI and *Xho*I restriction enzymes (New England Biolabs). Digestion of the LipL32 gene was done with 43 µl of purified PCR product (concentration of 17.8 ng/µl), 5 µl CutSmart Buffer, 1 µl of *Eco*RI enzyme and 1 µl *Xho*I enzyme. For plasmid digestion, 2 µl pRSET-C (0.5 ng/µl) was added with 41 µl $dH_2O$, 5 µl CutSmart Buffer, 1 µl of *Eco*RI enzyme and 1 µl *Xho*I enzyme. Each digestion reaction was then incubated at 37 °C for 1 hour. Digestion products were electrophoresed on 1% agarose to separate digested LipL32 and pRSET from buffer components and restriction enzymes. It was then purified using a gel extraction kit (Qiagen), the concentration was measured by Nanodrop, and followed by ligation. The digestion product of the pRSET-C plasmid and LipL32 gene was ligated using the T4 DNA ligase kit (New England Biolabs) by adding 50 ng of pRSET, 38 ng of LipL32gene, 2 µl ligation buffer, 1 µl ligase enzyme and sterile $dH_2O$ up to a volume of 20 ml. This reaction was gently mixed by tapping the tube, and then incubated overnight at 16°C. The process was then continued with transformation into *E. coli* cells.

### *2.3. Transformation of plasmid pRSET-LipL32 into E. coli competent cells*

In this study, transformation of recombinant plasmid pRSET-LipL32 was performed using competent *E. coli* BL21 (DE3) pLysS cells (Invitrogen). In this process, 5 µl ligation reaction of pRSET-LipL32 was added to 50 µl *E. coli* competent cells and incubated for 30 minutes in an ice bath. It was followed by a heat shock for 45 seconds at 42°C. The reaction was then immediately returned to the ice bath and incubated for 2 more minutes. Then 1 ml SOC media was added to the cells and incubated for 45 minutes at 37°C with shaking. This culture was spread on a SOB plate containing ampicillin (50 µg/ml) and chloramphenicol (35 µg/ml), and it was incubated overnight at 37°C. Colonies were then screened with PCR using forward primer LipL32-F and reverse primer LipL32-R2. A single positive colony was propagated for protein expression. Plasmid purification was also performed and sent to 1$^{st}$ base (http://www.base-asia.com/dna_sequencing/) for sequencing using forward primer T7 promoter and reverse primer T7 terminator and it was analysed using Geneious software (results not shown).

### *2.4. Protein expression and purification*

Expression of recombinant LipL32 was performed by propagation of *E. coli* BL21 cells containing plasmid-pRSET-C-LipL32. 10 ml SOB medium containing ampicillin (50 µg/ml) and chloramphenicol (35 µg/ml) was inoculated with a single colony and then incubated overnight at 37°C. The overnight culture was then added to 250 ml of SOB medium containing ampicillin (50 µg/ml) and chloramphenicol (35 µg/ml) and incubated for 4 hours at 37°C. After reaching an optical density (OD600) of 0.4-0.6, the cells were induced using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to express recombinant protein LipL32. The incubation was then continued for the next 4 hours at 37°C. The cells were harvested by centrifugation at a speed of 5000 rpm for 10 minutes and the supernatant was discarded. Cell pellets were stored at -20°C until needed for protein purification.

Purification of recombinant LipL32 in this study was performed using Ni-NTA Fast Start (Qiagen). The cell pellet was resuspended in 10 ml native lysis buffer containing lysozyme and benzonase nuclease, and then incubated in an ice bath for 30 minutes. The mixture was centrifuged at 10.000 x g for 30 minutes and the supernatant was collected. It was then loaded on a “Fast start column” containing Ni-NTA resin. The column was washed three times

using native wash buffer, and the recombinant protein was eluted in 2 ml fractions using native elution buffer. The size and purity of the recombinant LipL32 protein was analysed on 10% SDS PAGE. The concentration of purified Lipl32 protein was determined using the Bradford assay and then stored at -20°C.

### *2.5. Microagglutination test (MAT)*

Bacterial *Leptospira interrogans* strains were obtained from Kit Biomedical Research Netherland. The panel of 14 *Leptospira* serovars used for MAT in this study included Icterohaemorrhagiae, Javanica, Celledoni, Canicola, Ballum, Pyrogens, Cynopteri, Rachmati, Australis, Pomona, Grippotyphosa, Hardjo, Bataviae, and Tarrasovi.

To prepare the antigen for MAT, each *Leptospira* strain was cultivated in liquid EMJH medium and incubated at 30°C for 7 days until a density of $2x10^8$ *Leptospira*/ml was reached. Then 50 µl of this antigen was mixed with 50 µl diluted bovine serum (1:50, 1: 200, 1: 800, 1: 3200) and incubated at 30°C for 2 hours. The mixture of antigen-serum was then transferred to a glass slide and read with a phase contrast microscope at 100x magnification. The end points of the reading were determined by the occurrence of 50% or more agglutination (estimated from the amount of free *Leptospira*, which is about 50% or less) and the titer is defined as the highest serum dilution in serum-antigen mixtures that show 50% agglutination or more. Bovine sera from the slaughterhouse were screened in this MAT to find sera positive to Leptospirosis. These positive sera in MAT were then used in western blotting and ELISA for analysis of recombinant LipL32 protein.

### *2.6. Western blotting*

After SDS-PAGE analysis, purified recombinant protein was then transferred into PVDF membrane. Unspecific binding to the PVDF membrane was blocked by overnight incubation in 5% skim milk in PBS at 4°C. Then, incubation was continued with bovine serum 1:100 diluted in PBS for 90 minutes at room temperature. The membrane was washed three times with PBS-Tween 20 (0.05%) and incubated with horseradish peroxidase – goat – anti bovine IgG - serum 1:2500 diluted in PBS for 90 minutes at room temperature. The membrane was washed three times with PBS Tween 20 (0.05%) and the presence of the antigen-antibody reaction was detected by adding 3,3'-diaminobenzidine in 50 mM Tris-HCL, pH 8.

### *2.7. Enzyme-linked immunosorbent assay (ELISA)*

Purified LipL32 recombinant protein was diluted to a final concentration of 10 µg/ml in coating buffer (carbonate-bicarbonate, pH 9.6) and 100 µl of this antigen was loaded in the wells of a Maxisorb NUNC plate and then incubated overnight at 4°C. The plate was then washed using PBS-Tween20 (0.05%). Nonspecific binding to the plate was blocked by incubation with 150 µl blocking buffer (5% skim milk in PBS) for 2 hours at room temperature. The plate then washed three times using PBS-Tween20 (0.05%), 100 µl of bovine serum diluted 1/100 in PBS was added, and the plate was incubated for 1 hour at room temperature. Next, the plate was washed four times using PBS-Tween20 (0.05%), and 100 µl horseradish peroxidase – goat – anti bovine IgG – serum diluted in 1:2500 in PBS was added, and the plate was incubated for 1 hour at room temperature. The plate was washed four times using PBS-Tween20 (0.05%), 100 µl ABTS in substrate buffer was added, and the plate was incubated for 5-10 minutes at room temperature with shaking. The absorbance was read with an ELISA reader at a wavelength of 405 nm.

## 3. Results and discussion

Recombinant proteins have been widely used as antigens for diagnostic tests, especially for zoonotic diseases such as Leptospirosis. Compared to the native protein, recombinant protein is safer because it does not require live infectious bacteria that can harm human health. Moreover, the production of recombinant protein is usually cheaper and easier.

Recombinant LipL32 protein has been reported as an immuno-dominant antigen in ELISA tests[10]. Many studies also indicated that ELISAs with LipL32 could be used as the initial screening test against *Leptospira* infection[14].

Therefore, in this study we produced recombinant LipL32 protein and use it as an antigen in an ELISA for the detection of Leptospirosis in Indonesia.

### *3.1. Protein expression and purification*

In this study we successfully produced recombinant LipL32 protein. From 500 ml culture we could obtain 2 ml purified protein in soluble form with a concentration of 1 mg/ml. Analysis of the recombinant LipL32 protein using SDS-PAGE (Fig. 1a) showed fractions of the first, second and third elution in lanes 5, 6 and 7 with a band at 32 kDa which is the correct size for LipL32 protein[12]. This result also demonstrated that expressed recombinant LipL32 protein has a degree of purity of more than 90%.

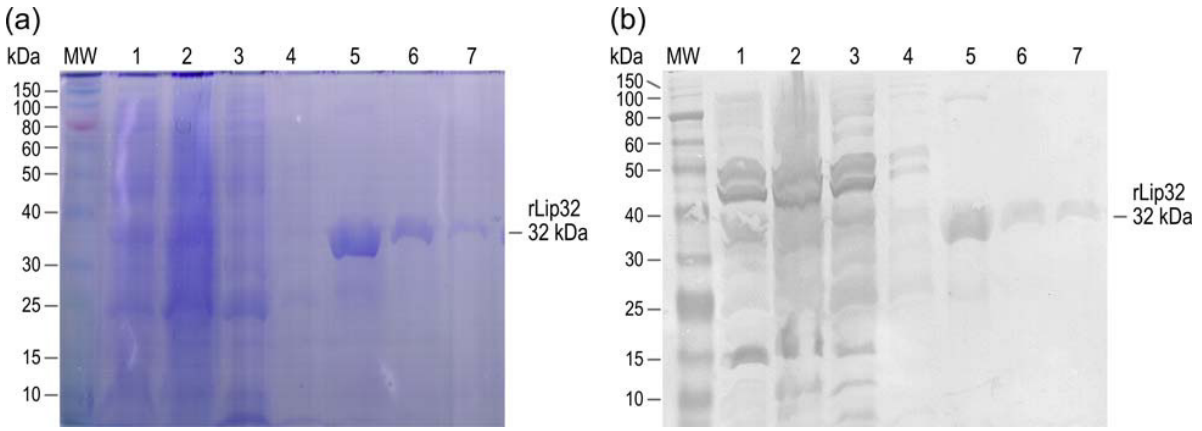


Fig. 1. Expression and purification of recombinant LipL32 protein (rLip32). The analysis shows (a) SDS-PAGE 10%; (b) Western blot of rLipL32 with bovine serum that reacts positive for Leptospiorosis. Lanes 1 and 2 show results from expression at 0 and 4 hours after induction with IPTG. Lanes 3 and 4 are flow-through and wash buffer of the purification step. Lanes 5, 6, 7 show the protein present in the first, second and third fraction, respectively.

The pRSET-C vector used in this study is an expression vector with the T7 promoter and has a 6xHis tag located at the N-terminal. The His-tag was chosen because of its advantages over other fusion tags. A His-tag is a small tag consisting of only 6 amino acid residues that is capable of interacting with a "immobilized metal affinity chromatography" (IMAC) resin such as Ni(II)-nitrilo-triacetic acid (Ni-NTA). The use of Ni-NTA resin for the purification of proteins is “economic” as it can be regenerated for reuse. In addition, the recombinant protein also can be eluted under mild conditions by adding 100-250 mM imidazole, pH <5.0, or 10 mM EDTA[18]. Also, the use of the pRSET vector with His-tag made the purification process easy as the His-tag expressed on recombinant proteins will bind to the nickel ions of the affinity resin. In order to obtain recombinant protein with high purity it can be easily washed and separated from other whole cell proteins. In this study we only amplified the LipL32 gene that encodes amino acids 21 to 272 because the first 20 N-terminal amino acids of LipL32 code for a "signal peptide" which is hydrophobic and causes aggregation of expressed proteins[12,19].

In the present study functional analysis of recombinant LipL32 was performed using western blotting with bovine serum that was positive for Leptospirosis (previously were tested with MAT). Fig. 1b, lanes 5, 6 and 7 showed bands with a size of 32 kDa that indicated that recombinant LipL32 protein can be detected by bovine serum positive Leptospirosis. One of the advantages of the 6xHis-tag is its small size, so that it does not interfere with the interaction of recombinant protein antigens and antibodies.

### *3.2. Microagglutination test (MAT)*

In this this study, various bovine serum samples from the slaughterhouse were tested for seropositivity to Leptospirosis using MAT. Table 2 shows 12 positive and one negative serum selected on the basis of the MAT results. The MAT titer of bovine serum was in the range of 100 to 1600, and mostly positive to *Leptospira* serovar Hardjo (9 out of 12 sera). Some of these sera were also positive to serovars Tarrasovi, Icterohaemorrhagiae and

Bataviae. A previous study has reported that mammals like cattle and bovine has an important role as a reservoir host for *Leptospira*. Common serovars that infect cattle include Hardjo, Pomona, Grippotyphosa and Icterohaemorrhagiae, while other serovars such as Australis, Autumnalis, Bataviae, Bratislativa, Canicola, Hebdomadis, Kremastos, Sejroe and Tarassovi are less commonly found in cattle[1].

Table 2. MAT titer, western blotting and ELISA rLipL32

| Bovine serum | MAT titer | Western Blotting | ELISA LipL32 |
|---|---|---|---|
| Bovine 56 | Hardjo (1/400); Bataviae (1/100); Icterohaemorrhagiae (1/100) | Lane 1 (Negative) | 1.05 |
| Bovine 53 | Tarassovi (1/1600) | Lane 2 (Negative) | 0.51 |
| Bovine 52 | Icterohaemorrhagiae (1/100) | Lane 3 (Negative) | 0.57 |
| Bovine 48 | Hardjo (1/400) | Lane 4 (Positive) | 1.03 |
| Bovine 47 | Hardjo (1/100) | Lane 5 (Positive) | 0.91 |
| Bovine 42 | Hardjo (1/1600), Tarassovi (1/100) | Lane 6 (Positive) | 0.92 |
| Bovine 39 | Hardjo (1/400) | Lane 7 (Positive) | 1.87 |
| Bovine 31 | Tarassovi (1/400) | Lane 8 (Negative) | 1.06 |
| Bovine 22 | Hardjo (1/100) | Lane 9 (Positive) | 1.60 |
| Bovine 17 | Hardjo (1/400); Icterohaemorrhagiae (1/100) | Lane 10 (Positive) | 0.81 |
| Bovine 15 | Hardjo (1/400) | Lane 11 (Positive) | 1.37 |
| Bovine 12 | Hardjo (1/400) | Lane 12 (Negative) | 0.87 |
| Bovine 10 | 0 (negative) | Lane 13 (Negative) | 0.98 |
| FCS (-ve) | Not Done | Not Done | 0.08 |

### *3.3. Enzyme-linked immunosorbent assay (ELISA) and western blotting of recombinant protein LipL32*

In this study we performed ELISA tests using recombinant LipL32 protein as antigen in a final concentration of 10 μg/ml and tested 12 sera that were positive for Leptospirosis (Fig. 2). 1 negative bovine serum and 1 fetal calf serum (FCS) were also included in this ELISA as negative controls. Fig. 2 and Table 2 show that the positive sera mostly could detect LipL32 in the ELISAs, 6 sera with OD >1, 4 sera with OD between 0.8-0.9, and 2 other sera had very low OD with ODs of around 0.5. The bovine serum with a negative titer on MAT reacted in this ELISA with an OD of 0.98, but the OD from fetal calf serum (FCS) was low (0.08).

Previous studies using bovine serum showed that a LipL32-based ELISA has great potential as a serological test for detection of infection by pathogenic *Leptospira* with a relative sensitivity and specificity of 100% respectively compared with the MAT[15]. However, in our study we found high OD for bovine serum that was negative in MAT. Many reasons could cause this nonspecific binding including blocking process of ELISA that was not optimal and the quality of the bovine serum.

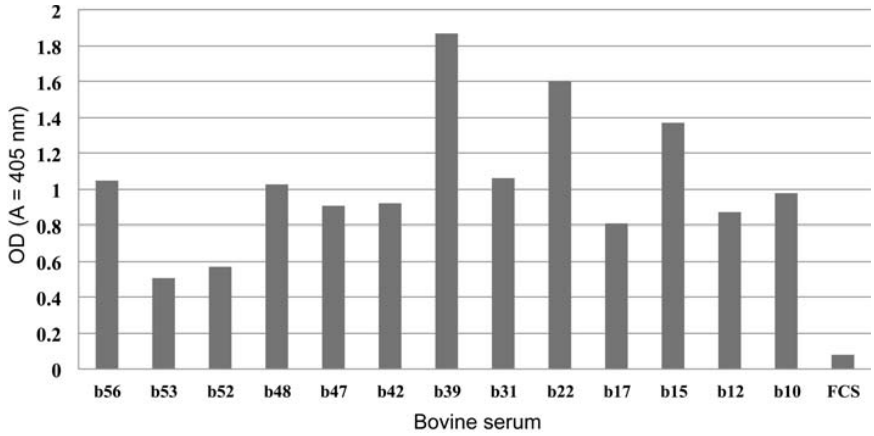


Fig. 2. ELISA of recombinant LipL32 protein, using 12 bovine serum positive Leptospirosis and one negative Leptospirosis.

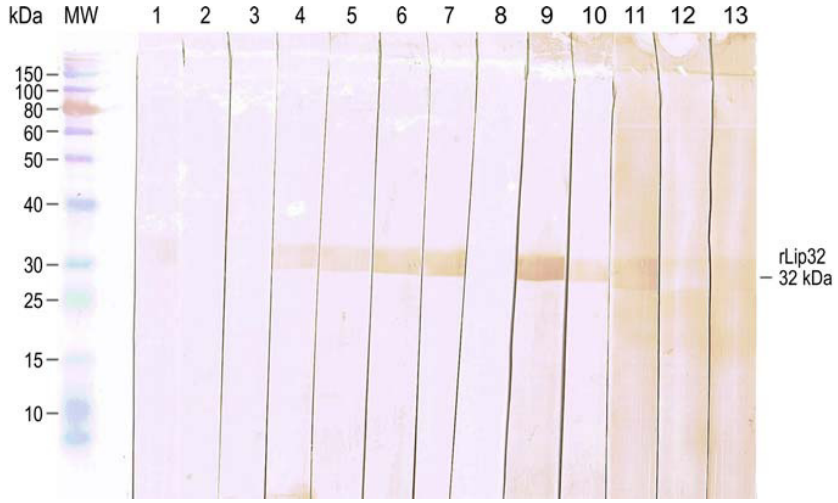


Fig. 3. Western blotting of rLipL32 using 12 bovine sera positive Leptospirosis (lane 1 to 12) and one negative Leptospirosis (lane 13).

Furthermore, these bovine sera were also analyzed using western blotting with recombinant LipL32 protein (Fig. 3) and showed bands with a size of 32 kDa in lanes 4, 5, 6, 7, 9, 10 and 11. This result demonstrates that most bovine sera were able to bind the LipL32 protein. The results from western blotting mostly correlate with the ELISA results (Table 2). High sensitivity and specificity of ELISA using protein LipL32 has been reported in previous studies[14,15], but not in this study. Therefore, a further analysis using a larger number of samples is required.

## 4. Conclusions

The results in this study show the potential of recombinant LipL32 protein as an antigen for Leptospirosis detection. Expressed recombinant LipL32 protein from *Leptospira pomona* could detect bovine serum positive MAT with serovar Hardjo and was consistent with previous studies reporting that LipL32 is capable of detecting a wide range of pathogenic *Leptospira*[12]. The number of serum samples analysed in this study is still very limited, and therefore the sensitivity and specificity can not be fully determined. Therefore, further studies of optimization and validation need to be be carried out regarding application of LipL32-based ELISA for Leptospirosis detection in Indonesia.

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